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THE RECOVERY OF PHYTOACTIN FROM WHITE PINE NEEDLES

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THE RECOVERY OF PHYTOACTIN FROM WHITE PINE NEEDLES

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INTRODUCTION

Ground and aerial applications of the antibiotic Phytoactin to western white pine (Pinus monticola) infected with Cronartium ribicola J. C. Fisch. ex. Rabenh. in Region 1 were initiated in 1958 and 1959, respectively. The recovery of Phytoactin from western white pine needles sprayed in 1959 was attempted in late 1960 through laboratory recovery techniques developed by Pabst Laboratories (currently known as P-L Biochemicals, Inc.). An extraction procedure for recovering Phytoactin from western white pine needles, including qualitative and quantitative measurement techniques was provided in 1962. However, a low degree of antibiotic recovery, estimated at 10 percent, was obtained by this technique. A 4-year research grant was placed by Pabst Laboratories with the Department of Plant Pathology at Washington State University in 1962 to develop better techniques for the isolation and measurement of Phytoactin from treated western white pine. A modified technique developed through this research project was provided Region 1 personnel in 1964 (1). The technique was adapted for production type processing of samples in the Region 1 laboratory in Spokane. A production schedule was achieved in late 1966 and continued into early 1967 to process samples collected in 1965 and 1966. A majority of the samples were collected from pole size western white pine stands included in the Phytoactin aerial spray program in northern Idaho. The remaining samples were collected from various antibiotic study plots in Region 1. Samples from untreated trees and study plots, collected in 1967, were processed in early 1969 in Missoula. The results for samples collected in 1965, 1966, and 1967 are presented in this report.

METHODS AND MATERIALS

The procedure in collecting samples consisted of stripping all the needles except juvenile growth from the branches, mixing the needles thoroughly, placing them in a cylindrical cardboard carton containing drierite (anhydrous calcium sulphate) for at least 6 weeks. After 6 weeks the needles were removed and ground in a Wiley Mill to a 40-mesh particle size. The ground material was placed in a sealed glass jar ready for extraction. The extraction procedure developed at WSU was used for all samples (exhibit A). All samples were processed the same way with a minimum degree of variation.

In many instances the extracts were stored for extended periods, up to a year, in a dry state following the millipore filtration step. The filters, when placed in the beakers for resuspension, were carefully positioned along the

sides to facilitate the elution of the residues. The residue was assayed for biological activity by the paper disc-agar plate method using conidial suspensions of Glomerella cingulata (Ston.) Spauld. and Schrenk (= Colletotrichum gloeosporioides Penz.) as the test organism. The bioassay method is the only known quantitative measure of Phytoactin. The bioassay methodology described by Stoner (8) was used with the following exceptions. The inoculum was propagated by shake culture in a peptone potato extract medium consisting of 10 percent potato extract, 0.5 percent peptone, and 1 percent glucose per liter of distilled water. Spore concentration varied somewhat between each bioassay series ranging between 150,000 and 200,000 spores per milliliter of seeded agar. A 10 ml. quantity of seeded agar was pipetted into each Petri plate. The poured plates were not stored as suggested in sealed plastic bags. Inhibition zones were measured to the nearest 0.5 millimeter on a Fischer-Lilly Antibiotic Zone reader after a 24- to 36-hour incubation period.

RESULTS

A total of 281 identified needle samples, collected in 1965 and 1966 from western white pine stands included in the aerial spray program, were processed. Nineteen samples were from untreated trees. Twenty-one percent of these showed positive or inhibitory activity against G. cingulata in the bioassays. Thirty-seven percent of the 262 samples from Phytoactin-treated trees showed positive activity.

Numerous needle samples were collected from study plots. Positive activity was obtained from untreated trees. All of the study plot samples, except those from the Jordan Creek helicopter test plots and the whitebark pine (Pinus albicaulis) studies, were from western white pine less than 20 years old. Positive activity was also obtained from needles of untreated whitebark pine.

Needles collected from the Jordan Creek helicopter test plots the same year of treatment, 1965, and 2 years later, 1967, were analyzed. The test series consisted of eight 40-acre blocks including two untreated blocks. One composite needle sample, representing several trees, was collected from each block in 1965. Half, or 50 percent of the samples, including one untreated block, showed positive activity. Six samples representing three trees in each block were collected in 1967. Eight of the 12 samples from the untreated blocks, or 75 percent, showed positive activity. Eleven of the 36 samples from the treated blocks, or 31 percent, showed positive activity. The average inhibition was nearly six times greater in the untreated samples than in treated samples. The results of the 1967 samples, when summarized by test block, or comparable to the 1965 sample base, showed 88 percent with positive activity, including the two untreated blocks.

Since a reasonable doubt existed concerning the positive activity for untreated tissue extracts, a special study was undertaken in 1967. Three trees from two age classes (15- and 40-year-old western white pine) in each of two Forests (St. Joe and Clearwater), or a total of 12 trees were sampled from areas with no known history of antibiotic treatment. Four of the twelve samples, or 33

percent, showed positive activity in the bioassays. Three of the four positive samples were from 15-year-old white pine in the St. Joe Forest.

DISCUSSION

A study of the attempted recovery and measurement of Phytoactin from numerous samples of white pine needles collected from widely scattered areas in northern Idaho and western Montana showed highly variable and inconsistent results. A significant amount of growth inhibition of G. cingulata by extracts from some of the untreated trees was discovered in the bioassays only after a great many needle samples had been analyzed. Needle samples collected from study plots involving reproduction and sapling size white pine showed the greatest amount of inhibitory activity. Investigation of the interference phenomenon showed a similar degree of inhibition activity from needles of 15- and 40-year-old western white pine with no known history of antibiotic treatment. Inhibition zones of unpredictable occurrence and intensity from untreated tissue, although not identical to the zones produced by Phytoactin standard solutions, prevented any meaningful measurement of recovered antibiotic. Noncharacteristic inhibition zones from untreated tissues observed in the recovery of Phytoactin from whitebark pine were not reported to interfere with measurements of antibiotic activity (3). Reports of other procedures for recovering Phytoactin have not indicated an interference phenomenon (2, 4, 5, and 7). In addition, interference or masking of activity was not found for abiotic compounds from untreated western white pine tissue in preliminary extracts (1, 5). However, the recovery procedures developed at Washington State University could extract proteinaceous and possibly other type compounds, in addition to Phytoactin, inhibitory to G. cingulata (6).

The modified technique did not include provisions for further purification of the antibiotic in the extraction procedures. Additional purification steps were not used because they were not considered practical on a production basis or even necessary.

One major difference in the bioassay methodology employed in the preliminary extractions (1, 3) and by Region 1 personnel was spore concentration in the seeded agar. An inversely proportional relationship between spore concentration and size of inhibition zone determines the sensitivity of the bioassay. The spore concentrations used in the preliminary extractions were over three times that used by Region 1 personnel. Noncharacteristic inhibition zones become more significant with (1) lower spore concentrations, and (2) a greater number of samples analyzed.

CONCLUSIONS

The techniques for the recovery and measurement of Phytoactin from western white pine needles used by Region 1 personnel to date have not produced reliable results. Interference by an unknown identity from the needles obscured any meaningful measurement of antibiotic recovery in a standardized bioassay procedure utilizing G. cingulata. Additional laboratory investigation

is necessary to replace or improve the existing techniques if reliable results are to be obtained. The evidence shows some of the difficulties that may be encountered when preliminary research results are adapted for project work.

EXHIBIT A

PHYTOACTIN EXTRACTION PROCEDURES
FOR DRIED PINE NEEDLES

Step 1. Petroleum ether cleaning

Add 30 grams of ground, dried pine tissue to a 300-ml. erlenmeyer flask and cover tissue with petroleum ether. Stir sample with magna stirrer for 15 minutes. After settling, decant petroleum ether and discard. Add additional petroleum ether and agitate for 30 minutes with magna stirrer. Transfer contents to sintered glass filter and remove petroleum ether under vacuum. Discard petroleum ether. After the pine tissue is completely dry, weigh out 25 grams and add to clean 200-ml. erlenmeyer flask. Transfer identifying label to this flask.

Step 2. Acetone extraction

Add 100 ml. of acetone to pine tissue and extract for 30 minutes on magna stirrer. After settling, decant and collect acetone in a 125 ml. flask with identifying label. Add an additional 100 ml. of acetone to pine tissue and extract with magna stirrer for an additional 30 minutes. Combine acetone extractions and draw acetone solvent through sintered glass filter and collect acetone extract below in clean 200-ml. flask. Rinse the extraction flask with 10 ml. of acetone and add to sintered glass filter. Transfer identifying label to acetone extraction, place in water bath hood, and evaporate to dryness.

Step 3. Millipore filter operation

To dry residue in 200-ml. flask, add 15 ml. of methylene chloride to dissolve residue. Allow to set 45 to 60 minutes. After residue has dissolved, pour into cold 400-ml. beaker. Rinse flask with additional 10 ml. of methylene chloride. Prepare freezer cold millipore filter apparatus for vacuum filtering. Add 250 ml. of cold petroleum ether to 25 ml. of dissolved methylene chloride residue in 400-ml. beaker. Immediately pour contents of beaker into millipore filter apparatus. Draw solvent mixture through millipore filter with a strong vacuum. Use additional filters if first filter becomes clogged. Place filters in envelope and staple identifying tag to envelope. Store filters for 18 to 24 hours before continuing to next step.

Step 4.

Place millipore filter or filters in 20-ml. beaker containing 5 ml. of methanol. Wash residue from filters by swirling. Decant methanol into clean glass vial with identifying label attached. Evaporate to dryness in water bath hood. Resuspend solids in 1 ml. methanol-trito solution. Sample is now ready for bioassay with Glomerella cingulata.

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